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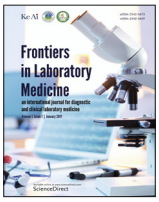

Relationship between oxidative stress in patients with HBV-induced liver disease and HBV genotype/drug-resistant mutation

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ABSTRACT

Objective: To explore the correlation of oxidative stress in patients with chronic hepatitis B and degree of severity of the disease with HBV genotype and drug-resistant mutation.
Methods: A total of 296 patients with diagnosed chronic hepatitis B were selected from Febuary 2014 to April 2016 in Mianyang Central Hospital, including 145 cases of chronic hepatitis B (CHB), 101 cases of hepatitis B cirrhosis (HBC), and 50 cases of hepatocellular carcinoma (HCC). Three HBV genotypes (B, C and D) and eight drug-resistant mutation genes (rt180L, rt204M, rt207V, rt236N, rt250M, rt181A, rt184T and rt202S) were detected by PCR-reverse dot blot method. In addition, total oxidative stress (TOS), and total antioxidant status (TAS) were measured, on the basis of which oxidative stress index (OSI) was calculated. Furthermore, the differences of TOS, TAS and OSI levels were compared between different liver diseases, different genotypes or drug-resistant mutation, and also the correlations were analyzed between HBV genotype, drug-resistant mutation, patient's oxidative stress status and disease severity.
Results: Serum TOS and OSI levels, HBV-B/C ratios and drug-resistant mutation rates increased gradually with the severity of liver disease (CHB < HBC<HCC, $P < 0.05$). Serum TAS levels decreased with degree of severity of the disease, but there was no statistical difference between CHB group and HCC group. Except TAS levels in patients at PHC group, compared with patients without mutation in HBV, the patients with drug-resistant mutation had higher TOS and OSI levels, but lower serum TAS levels ($P < 0.05$). Drug-resistant mutation rate was positively correlated with TOS ($r = 0.476$, $P < 0.001$) and OSI ($r = 0.441$, $P < 0.001$) levels, but negatively correlated with TAS level ($r = -0.249$, $P < 0.001$), except TAS level in patients at PHC group. In addition, the number of mutation sites was positively correlated with disease severity ($\gamma = 0.614$, $P < 0.001$).
Conclusions: There are different degrees of oxidative damage in patients with HBV-induced liver disease, and the degree of the damage depends on HBV genotypes and drug-resistant mutations. Therefore, oxidative stress parameters might be useful indicators of progression of HBV-induced liver disease in patients.

Introduction

According to a 2013 epidemiological survey, there are approximately 100 million HBV carriers in China,[1] including patients with chronic hepatitis B (CHB), post-hepatitic cirrhosis, which is referred to as hepatitis B cirrhosis (HBC) in this paper, and hepatocellular carcinoma (HCC). Some studies indicated that almost 60% of HBC cases and 80% of HCC cases are caused by continuous HBV infection,[2] which is also the main cause of HBC and HCC cases in China. According to the World Health Organization (WHO), a total of 650,000 people die of liver failure, HBC, and HCC caused by HBV infection worldwide annually.[3] Many studies have shown that the HBV gene mutation is closely related to the risk of HCC;[4–6] until now, however, the relationship between the HBV genome and occurrence and development of HBC and HCC remains unclear.[7] In China, detection of the HBV genotype and mutant genes causing drug resistance is widely applied to guide the use of antiviral drugs for HBV-induced liver disease. However, there are few reports about whether mutant genes causing drug

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resistance of HBV correlate with risk and development (degree of severity) of HBC and HCC and oxidative stress (OxS) in patients.

OxS refers to a disease status caused by an imbalance between oxidation and antioxidation in the body, which is due to excessive consumption of antioxidants or an excessive production of oxidants.[8] Thus, OxS is closely related to inflammatory response.[9] However, there are few reports about whether OxS correlates with the HBV genotype and mutant genes causing drug resistance at different stages of HBV-induced liver disease.

On the basis of the above facts, this paper determined HBV genotypes, drug resistance mutation rates, serum levels of total oxidative stress (TOS), and total antioxidant status (TAS), and calculated the oxidative stress index (OSI) at different stages of HBV-induced liver disease. Furthermore, we explored the correlation of the HBV genotype and drug resistance mutation rate with OxS in order to understand the relationship of the drug resistance mutation rate with OxS and the degree of severity of the disease after patients were infected with different HBV genotypes.

Materials and methods

Enrollment of patients

Between February 2014 and April 2016, a total of 296 cases (232 male and 64 female; mean age: 42.4 ± 13.5 years) in Mianyang Central Hospital (Mianyang, China) were enrolled in the study. They were diagnosed with HBV-induced liver disease through the detection of serological markers of HBV infection and/or HBV DNA. CHB patients were defined as those with persistent hepatitis B surface antigen and/or elevated HBV DNA levels for six months or more.[3] Among them, there were 145 cases of CHB, 101 cases of HBC, and 50 cases of HCC. Diagnoses of all of the cases were in accordance with WHO 2015 criteria for CHB,[3] the Asian Pacific Association for the Study of the Liver (APASL) 2012 criteria for liver fibrosis,[10] and the National Comprehensive Cancer Network (NCCN) 2016 criteria for hepatobiliary tumors.[11] In order to enhance the credibility of the results, we excluded diseases that may lead to increased TOS and / or TAS decline, such as diabetes, cardiovascular disease, other tumors, pulmonary infection, gastrosis, nephrosis, pancreatitis and neurological disease, and so on.

Methods

Sampling

Two Vacutainer® blood collection tubes (Becton Dickinson, USA) without additives were used to collect blood samples from each patient. Each tube contained approximately 5 mL of blood. After clots shrunk naturally, the blood sample was centrifuged at 3000 r/min for 10 min and serum was separated. Serum in 1 tube was used for the determination of serum TOS and TAS levels within 4 h. Serum in the another tube was used for the detection of the HBV genotype and mutant genes causing drug resistance according to the manufacturer's instructions. This tube containing serum was stored at −20 °C until use.

Measurement of OxS parameters

OxS parameters, including serum TAS and TOS levels, were determined using a 7600-020 automatic biochemical analyzer (Hitachi, Japan). One week before detection of OxS parameters for all patients, antioxidant drugs, such as spirulina, vitamin E, vitamin C, polyene, inosine tablets, coenzyme Q10, etc., were discontinued. In addition, refreshing drinks, including teas and coffees and their extracts, were also stopped.

Serum TAS level was determined by a modification of the method described by Erel O.[12,13] This assay relied on the ability of antioxidants in the sample to inhibit ABTS (2,2′-azino-di-3-ethyl benz-thiazoline sulfonate) from being oxidized into ABTS+ by a peroxidase metmyoglobin. An antioxidant with a known concentration (1.65 mmol/L) was used as the standard to calculate antioxidant levels in the samples. The TAS level was expressed as mmol Trolox equivalent/L (mmol Trolox equiv/L).

The serum TOS level was measured by a modification of Erel's TOS method,[13,14] which relied on the oxidation of ferrous ions into ferric ions in the presence of various oxidative species in an acidic medium. Ferric ion concentrations were measured by xylenol orange. The assay was calibrated with a standard hydrogen peroxide solution (39.16 μmol/L). Results are expressed as μmol H_2O_2 equivalent/L (μmol H_2O_2 equiv/L).

The TOS/TAS ratio was defined as OSI, which was calculated as follows:[13]

$$OSI[\text{arbitrary unit(AU)}] = \frac{TOS(\mu\text{mol } H_2O_2 \text{ Eq./L})}{TAS(\mu\text{mol Trolox Eq./L})} \times 100.$$

Detection of HBV genotype and mutant genes causing drug resistance

After DNAs of serum samples and positive and negative controls were extracted and purified by viral DNA extraction and purification kits (centrifugal column method, Yaneng Bio Co., Ltd., Shenzhen, China), PCR-reverse dot-blot hybridization was used to detect 3 HBV genotypes (B, C, and D) and 8 mutant genes causing drug resistance (t180L, rt204M, rt207V, rt236N, rt250M, rt181A, rt184T, and rt202S). The 7500 Real-time PCR System (Applied Biosystems, Foster City, CA, USA) was used.

Statistical analysis

One-sample Kolmogorov–Smirnov test was used for the normality test of measurement data and results are expressed as $\bar{x}\pm s$ or M (min, max). Enumeration data is expressed as n or n (%). In order to analyze the difference among groups, (1) Kruskal–Wallis test (non-normally distributed data) or single factor analysis of variance (normally distributed data) was used to identify the difference among multiple groups of measurement data; (2) two-sample Kolmogorov–Smirnov test (non-normally distributed data) or Least Significant Difference (LSD) analysis of variance (normally distributed data) was used to identify the difference between every 2 groups of measurement data; (3) contingency table χ^2 test was used to identify the difference among multiple groups of enumeration data; and (4) continuity-corrected χ^2 test was used to identify the difference between every 2 groups of enumeration data. Correlation was analyzed using Goodman and Kruskal's gamma or Spearman's rho. SPSS 19.0 (SPSS Inc., Somers, NY, USA) and MedCalc 12.7 software (MedCalc Software, Mariakerke, Belgium) were used to statistically analyze the data, and $P < 0.05$ was taken as statistically significant.

Results

Results of patients at different stages of HBV-induced liver disease

Results of HBV genotypes, drug resistance mutations, and OxS in patients at different stages of liver disease are listed in Table 1. It can be seen that there were statistical differences in gender, age, OxS parameters, HBV genotype distribution, and drug resistance mutations among patients at different stages of HBV-induced liver disease ($P < 0.05$).